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Role of Gonadotropin-Releasing Hormone Pulse Frequency in Differential Regulation of Gonadotropins in the Gilt

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ABSTRACT

We tested the hypothesis that different GnRH pulse frequencies will affect serum LH and FSH differently. Ovariectomized gilts (n = 6), immunized against GnRH, were given 200-ng pulses of GnRH agonist (GnRH-A) every 180 min for 3 days (pretreatment), followed by GnRH-A pulses every 30, 60, or 180 min for 3 days (treatment) in a Latin rectangle design. Mean gonadotropin concentrations did not change over time when GnRH pulses were administered every 180 min. Initiation of high GnRH-A pulse frequency (30 min) caused a robust increase in serum LH to 265% of the pretreatment level ($p \leq 0.007$) and a more moderate increase in serum FSH to 127% of pretreatment level ($p \leq 0.02$). After 66 h of frequent pulsing, desensitization had occurred and serum LH concentrations were similar to pretreatment concentrations, but serum FSH had decreased to 53% of pretreatment levels ($p \leq 0.0008$). After 72 h of treatment, 5 μg GnRH-A was infused to estimate residual releasable pools of LH and FSH, and the amounts of LH and FSH released were negatively correlated with GnRH-A pulse frequency. The results of this study imply that the LH surge is terminated because the pituitary gland becomes incapable of responding to an otherwise adequate stimulus, and not because of exhaustion of releasable LH pools. Our results confirm that in the pig the response to altered GnRH-A pulse frequency differs between LH and FSH. High GnRH pulse frequency is more effective in acutely releasing LH than FSH. Low pulse frequency of GnRH supports FSH synthesis and release, but is not as effective in increasing LH concentrations, while high GnRH pulse frequency inhibits FSH synthesis and release.

INTRODUCTION

LH and FSH are secreted differentially during the estrous cycle [1] and during several experimental situations [2]. Several factors, such as steroids and gonadal peptides [3, 4], affect LH and FSH concentrations, but GnRH has been accepted as the only hypothalamic releasing factor responsible for LH and FSH synthesis and release. The question arises whether GnRH plays a primary role in differential regulation of LH and FSH.

Pulse frequency of GnRH [5, 6] and LH [7, 8] is known to change throughout the estrous cycle and postpartum period. In several species such as rats [9–11], monkeys [12], cattle [13], and sheep [2, 14], differential release of LH and FSH, as well as differential expression of LHβ-, FSHβ-, and α-subunit mRNA, has been shown to be associated with different patterns of GnRH acting upon the pituitary gonadotrophs. High GnRH pulse frequencies tend to favor LH production and release while low frequencies favor FSH [10, 11]. Only a minimal amount of work of this nature has been performed and reported in the pig, undoubtedly one of the more important livestock species and an important model for biomedical research.

To study the influence of GnRH pulse frequency on LH and FSH release, the pituitary has to be isolated from endogenous GnRH input, followed by GnRH replacement in a defined pattern. Initial work by Fraser's group [15] and our group [16] showed that neutralization of endogenous GnRH reduced serum LH and FSH, caused cessation of follicular development and the estrous cycle, blocked ovulation, and resulted in testicular atrophy. The immunization of gilts against GnRH specifically and effectively neutralizes GnRH, resulting in inhibition of its biological actions without affecting prolactin concentrations [16].

In the present study, we used ovariectomized gilts immunized against GnRH to gain further insight into the roles of GnRH pulse frequency on differential release of LH and FSH from the pig pituitary in vivo.

MATERIALS AND METHODS

Animals

Nine prepubertal crossbred gilts (Duroc × Hampshire; 144 days of age) were ovariectomized under general anesthesia to remove the effects of ovarian steroids and peptides on pituitary function, since we wanted to study the main effects of altered GnRH pulse frequency. This early age was chosen to create long-term ovariectomized gilts, so we could be reasonably sure that there were no longer any residual ovarian effects. At 201 days of age (Week 0 of experiment) gilts were immunized against GnRH to neutralize endogenous GnRH. Booster immunizations were given at Weeks 8 and 10. The six gilts with the highest anti-GnRH titers at Week 11 were used in the experiment. The Society for the Study of Reproduction guidelines ensuring the welfare and humane treatment of animals were observed, and the experiment was approved by the Institutional Animal Care and Use Committee.

Immunization

Synthetic GnRH acetate salt (Sigma Chemical Co., St. Louis, MO) was conjugated to BSA as described by Fraser et al. [17] and modified by Esbenshade and Britt [16]. Primary immunizations used 2.5 mg of GnRH conjugated to BSA per gilt, emulsified in 3 ml Freund's complete adjuvant (Sigma), and booster immunizations used 0.5 mg GnRH conjugated to BSA in 3 ml of Freund's incomplete adjuvant.

Experimental Design; Schedule for Treatment and Blood Sampling

Prior to each immunization, gilts were bled by jugular venipuncture. All blood samples were refrigerated (4°C) for 12–24 h before serum was separated by centrifugation (1700 × *g*; 30 min). Serum was kept frozen (−20°C) until

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assayed for LH, FSH, and GnRH antibody. Gilts were fitted with bilateral indwelling vena cava catheters [18] at the end of Week 12 (Day 0). One catheter was used for blood sampling, the other for infusion of GnRH agonist (GnRH-A).

The experiment was designed to determine whether different GnRH pulse frequencies would induce differential release of LH and FSH in the gilt. In the female pig, LH pulses are observed every 3–6 h during the luteal phase and every 1–2 h during the follicular phase [7, 19]. Therefore, gilts (n = 6) were treated with three different GnRH-A pulse frequencies: high, intermediate, and low (30-, 60-, or 180-min intervals). Gilts were randomly assigned to a Latin rectangle design with six columns representing gilts and three rows representing experimental periods. Each gilt received each treatment but in different experimental periods (2 gilts per treatment in each experimental period). Each experimental period lasted 7 days; details are depicted in Figure 1. Gilts did not receive any GnRH-A for 3 days between experimental periods.

A large dose of 5 μg GnRH-A (Bolus1) was administered initially with the intention of reducing variation among gilts by releasing readily releasable pituitary pools of LH and FSH before the initiation of the pulsing regimen. During the pretreatment phase each gilt received GnRH-A pulses every 180 min for 3 days because absence of GnRH stimulation causes down-regulation of GnRH receptors whereas intermittent administration of GnRH or GnRH analogues can up-regulate pituitary GnRH receptors and stimulate gonadotropin synthesis in the rat [20] and sheep [21]. GnRH receptor concentrations have not been reported in the pig, but GnRH-A pulses (100 ng every 2 h for 3 days; same agonist as in the present study) administered to GnRH-immunized gilts resulted in detectable LH in 50% of the samples [22]. The large GnRH-A dose at the end of each experimental period (Bolus2) was administered to determine residual releasable gonadotropin pools that remained after implementation of the various GnRH-A pulsing regimens. Blood samples were taken immediately before the 5 μg GnRH-A infusion (Bolus2) and after 5, 10, and 20 min and every 20 min thereafter until 4 h after the infusion.

GnRH-A

The GnRH agonist, d-Ala^6, des Gly-NH$_2$^{10} ethylamide GnRH (Sigma) was used to stimulate gonadotropin release in the GnRH-immunized gilts. During the pretreatment and treatment phases, gilts received 200 ng GnRH-A in 84-μl sodium citrate (3.5%, pH 7.4) as instant pulses (i.v.; over 5 sec) using Auto Syringe infusion pumps (Travenol; Hooksett, NH). During blood sampling intervals, pumps were disconnected and gilts were pulsed manually with 200 ng GnRH-A in 1 ml. The 5 μg GnRH-A infusions (Bolus1 and Bolus2) were administered in a volume of 2.1 ml over a period of 30 sec.

Hormone Assays and Antibody Titers

Serum LH and FSH were measured by RIA. All samples from a gilt were analyzed in the same assay. The intra- and interassay coefficients of variation at 1.7 ng/ml for the LH assays [23] were 7.8 and 23.3%. The average sensitivity was 0.2 ng/ml. For the FSH assay [16, 24], we used USDA–398–04P anti-porcine (p) FSH, [^{125}I]-USDA-pFSH-I–1 as the radiolabeled ligand, and USDA-pFSH-B–1 for standards. The incubation time with first antibody was increased from 48 to 60 h, and after addition of tracer the

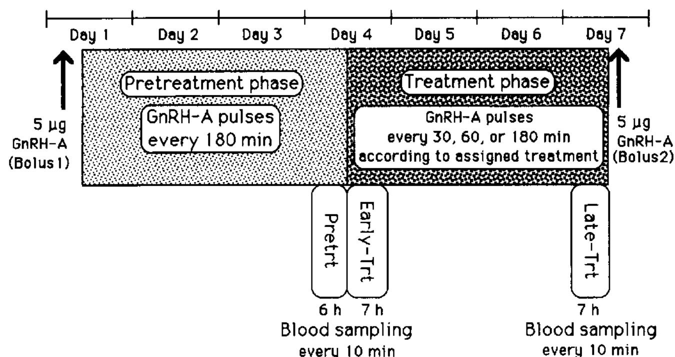

FIG. 1. Treatment and blood sampling schedule for one experimental period.

assay was incubated for an additional 48 h. The intra- and interassay coefficients of variation were 8.8 and 12.6% at 91 ng/ml, and 9.4 and 17.4% at 43 ng/ml. The average sensitivity was 12 ng/ml.

GnRH antibody titers were determined in GnRH binding assays based on the procedure of Esbenshade and Britt [16].

Statistical Analyses

Mean, baseline, and high hormone concentrations, defined as the average of all samples, the seven lowest samples, and the seven highest samples, respectively, were calculated for every sampling interval (pretreatment, early treatment, late treatment; Fig. 1) for each gilt during each experimental period. To justify comparisons between pretreatment (6-h sampling) and treatment (7-h sampling), only the first and the last 6 h of data were included for the early- and late-treatment sampling intervals, respectively, starting with one sample before GnRH administration. For baseline determination of early-treatment intervals, only data collected between 3 and 6 h were utilized to allow for stabilization of baseline after new treatments had been initiated. Undetectable hormone levels were allocated a value of 0.1 ng/ml for LH and 10 ng/ml for FSH.

To compare releasable pools of gonadotropins at the end of each experimental period, pituitary responses to GnRH-A were determined for the 4-h period after infusion of 5 μg GnRH-A by calculating the area under the LH and FSH response curves (AUC) above the pre-bolus baseline. Prebolus baseline was defined as the value of the sample taken immediately before the 5 μg GnRH-A application.

Data were subjected to least squares analyses of variance using the General Linear Models procedure of the Statistical Analysis System [25]. Each pig was used in each experimental period and each pig received each treatment. However, pigs received treatments in different order. Therefore, we checked first to determine whether a given treatment affected the subsequent treatment's effects. This step permitted us to separate the effects of time (experimental period) and type of previous treatment (30, 60, or 180). This analysis revealed no specific effects of previous GnRH-A pulse frequency. Experimental period was then used as a factor for further analysis of GnRH-A frequencies within sampling intervals (pretreatment, early treatment, late treatment) as required by standard Latin square analysis. This allowed us to account for variation introduced by pigs (columns) and the time of treatment during the experiment (experimental period; rows), and to detect variation introduced by type of treatment (GnRH-A pulse frequency). Data analysis among sampling intervals within GnRH-A

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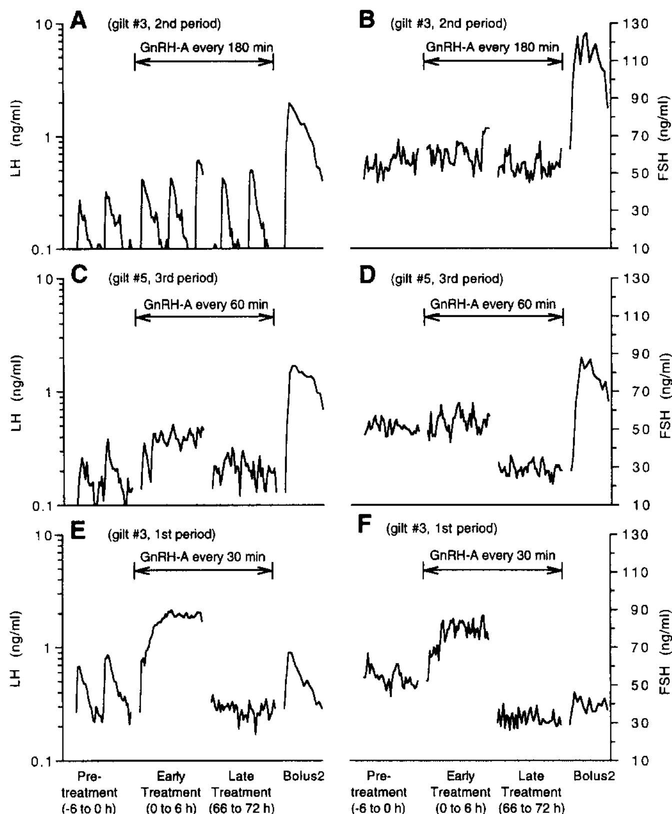

FIG. 2. Representative LH (**A, C, E**; log scale) and FSH (**B, D, F**) profiles of individual gilts. All gilts received 200-ng pulses of GnRH-A every 180 min for 3 days (pretreatment). Blood samples were taken every 10 min for the last 6 h of the 3-day pretreatment phase. Immediately thereafter, pulse frequency was changed to every 30 min (**E, F**) or 60 min (**C, D**), or gilts continued to be pulsed every 180 min (**A, B**) for 3 days. Blood samples were taken every 10 min for the first 7 h (early treatment) and the last 7 h (late treatment) of the 3-day treatment phase. Afterwards, gilts received 5 μg GnRH-A (Bolus2) and blood samples were collected for 4 h.

frequencies utilized a split-plot model for repeated measures with experimental period in the main plot and gilt within experimental period as the error term to test the main plot effect. Sampling interval and experimental period × sampling interval interaction were tested in the split plot. Means were compared by least significant difference or by preplanned orthogonal contrasts.

RESULTS

Effect of Immunization against GnRH

Serum concentrations of LH and FSH measured 1.5 ± 0.3 and 144 ± 7 ng/ml at Week 0 and were decreased by immunization against GnRH to 0.4 ± 0.2 and 65 ± 5 ng/ml ($p < 0.05$) before the initiation of the first experimental period at Week 12. Serum LH was at 0.2 ± 0.1 ng/ml before the initiation of the second and third experimental period, and serum FSH at 60 ± 5 ng/ml before the second and at 46 ± 3 ng/ml before the third experimental period. Anti-GnRH titers were elevated 2 wk after the first booster (Week 10), reached a maximum 1 wk after the second booster (Week 11), and stayed elevated during the application of the GnRH-A pulsing regimens.

Effect of GnRH-A Pulse Frequency

Pulsatility of LH and FSH was examined using visual appraisal and CLUSTER analysis [26], with both methods yielding the same results. During the pretreatment blood sampling interval, all gilts responded with a distinct LH pulse to each GnRH-A pulse (Fig. 2), whereas FSH pulses could not be detected consistently with either method. Gilts receiving GnRH-A pulses every 30 min during the treatment phase (Fig. 2, E and F) responded with a sharp increase of serum LH and FSH immediately after initiation of the more frequent pulsing regimen. Serum gonadotropins reached a plateau after 2–3 h and stayed at that level for the remainder of the early-treatment blood sampling interval. In these gilts mean LH increased to 265% of pretreatment concentrations ($p \leq 0.007$; from 0.33 to 0.86; SEM 0.09 ng/ml), and similarly, baseline LH increased to 423% ($p \leq 0.006$; from 0.22 to 0.93; SEM 0.12 ng/ml). Mean FSH increased to 127% ($p \leq 0.02$; from 43.3 to 54.8; SEM 2.4 ng/ml), and FSH baseline increased to 141% ($p \leq 0.007$; from 37.4 to 52.8; SEM 2.7 ng/ml). However, the 30-min GnRH-A pulse frequency was not able to maintain serum gonadotropins at those elevated levels. After 3 days of frequent pulsing (late-treatment sampling interval), mean LH had returned to 96% of pretreatment levels ($p > 0.9$; to 0.31 ± 0.09 ng/ml) and LH baseline to 114% ($p > 0.9$; to 0.25 ± 0.12 ng/ml), whereas mean FSH and baseline FSH concentrations in these pigs had decreased to 53% ($p \leq 0.001$; to 22.8 ± 2.4 ng/ml) and 49% ($p \leq 0.003$; to 18.2 ± 2.7 ng/ml) of pretreatment values.

Mean, baseline, and high LH differed among GnRH-A pulse frequencies during early treatment (Fig. 3), and were greater ($p < 0.05$) in gilts pulsed every 30 min than in gilts pulsed every 60 or 180 min. Mean and baseline LH during late treatment did not differ among GnRH-A pulse frequencies ($p > 0.1$), and concentrations in all treatment groups were similar to pretreatment concentrations ($p > 0.1$). In contrast, mean and baseline FSH were not different among GnRH-A pulse frequencies during early treatment but differed during late treatment and were lower ($p < 0.05$) in gilts pulsed every 30 or 60 min than in gilts pulsed every 180 min.

Effect of Experimental Period

Gilts receiving GnRH-A pulses every 30 or 60 min, i.e., gilts in which mean serum FSH concentrations during late treatment (25.5 ± 0.5 ng/ml) were decreased as compared to pretreatment concentrations (46.2 ± 0.6 ng/ml), had recovered FSH concentrations during pretreatment of the following experimental period (43.7 ± 0.5 ng/ml). However, LH concentrations in these gilts had not recovered and measured 0.32 ± 0.01 during pretreatment, 0.33 ± 0.01 during late treatment, and 0.22 ± 0.01 ng/ml during pretreatment of the following experimental period. Mean LH concentrations across all treatments were greater ($p \leq 0.05$) during the first experimental period than during later experimental periods. However, there were no indications of a sampling interval by experimental period interaction, and the percentage change from pretreatment to treatment concentrations was similar in all experimental periods (Table 1).

Response to the Final Challenge with GnRH-A

An inverse relationship existed between frequency of GnRH-A pulses during the treatment phase and AUC in response to the final administration of 5 μg GnRH-A (Fig. 4). FSH-AUC differed ($p \leq 0.05$) among the three treatment groups. LH-AUC, however, did not differ between the 30- and the 60-min group; but both groups responded with less LH release than the 180-min group ($p \leq 0.05$). In the

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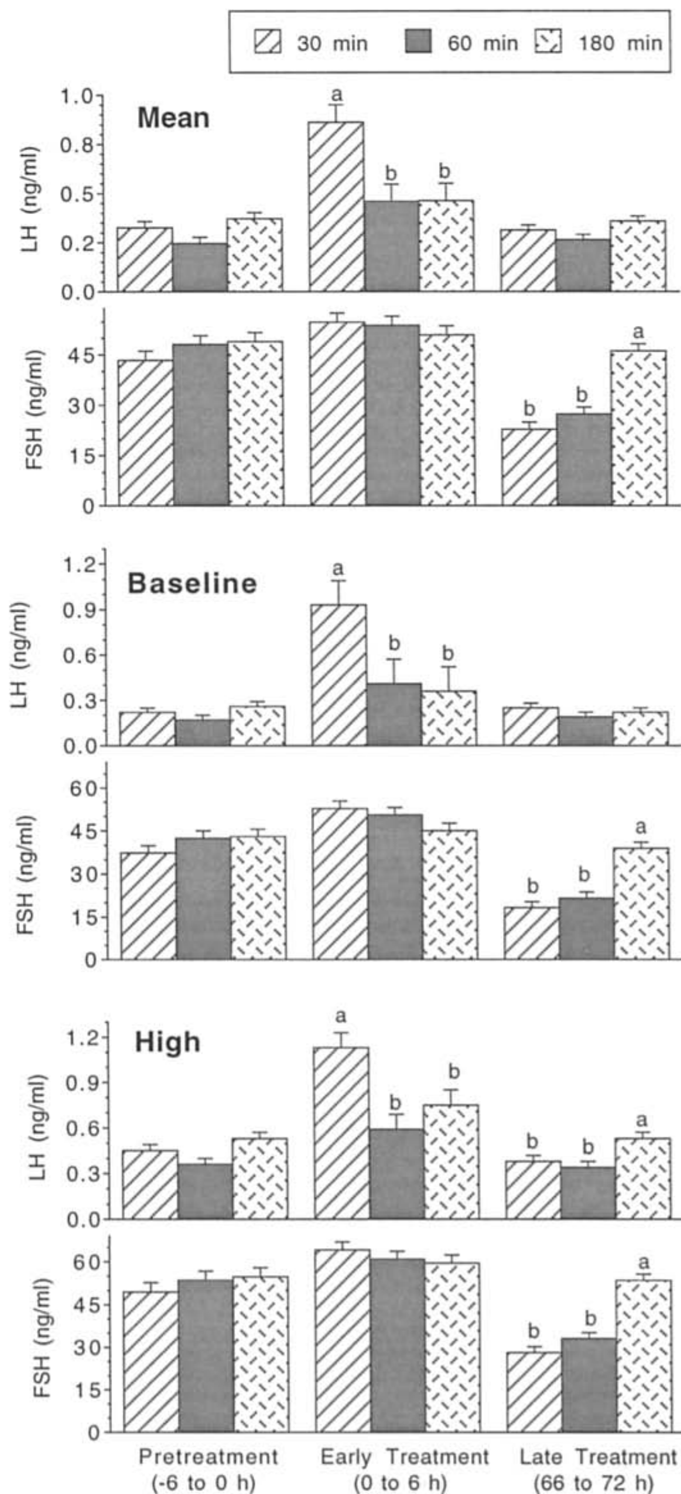

FIG. 3. Effect of GnRH-A pulse frequency on mean, baseline, and high gonadotropin concentrations at the end of the 3-day pretreatment phase (pretreatment) and the beginning (early treatment) and end (late treatment) of the 3-day treatment phase. Each column represents the least square mean (± SEM) of six gilts. Pretreatment hormone concentrations are shown to provide a reference value and are labeled according to the GnRH-A pulse frequency the gilts were going to receive during the following treatment phase. [a,b]Columns within sampling interval identified by different letters differ ($p \leq 0.05$).

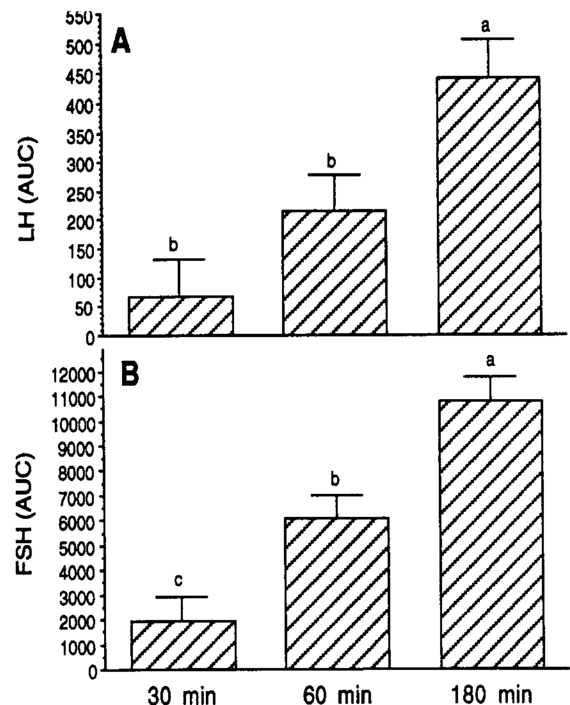

FIG. 4. AUC (ng × ml^{-1} × min) for serum LH (**A**) and FSH (**B**) after 5 μg GnRH-A (Bolus2) was given i.v. at the end of each treatment phase during which gilts had received GnRH-A pulses every 30, 60, or 180 min for 3 days. Columns (least square means ± SEM) within the same panel and identified by different letters are different ($p \leq 0.05$).

first experimental period, LH-AUC was greater ($p \leq 0.05$) than in the second or third experimental period (Table 2). There was no difference among experimental periods for FSH-AUC after the final 5 μg GnRH-A administration.

DISCUSSION

Effect of Frequency of GnRH Pulses

In a model in which the effects of endogenous GnRH were neutralized, we sought to determine whether different GnRH-A pulse frequencies altered the secretion of LH and FSH from the gilt pituitary gland in the absence of ovarian influences. We are not aware of other reports examining this question in the pig. The most indicative observations made were that 1) LH secretion was immediately and prominently affected by increased GnRH-A pulse frequency, but the effect of increased GnRH pulse frequency on FSH release was less pronounced, and 2) an effect of GnRH pulse frequency on FSH concentrations was not observed until the late-treatment sampling interval when high-frequency GnRH-A pulses resulted in reduced FSH concentrations.

In our model, the effects of different GnRH pulse frequencies on mean gonadotropin release paralleled effects on baseline concentrations. Observations from in vivo studies of lactating and weaned sows indicate that follicular development is not necessarily associated with a measurable change in mean gonadotropin concentrations [8, 27]. In those studies, basal LH concentrations and LH pulse frequency showed more positive correlations to follicular parameters than did mean LH concentrations, but there was considerable sow-to-sow variation. It seems important to recognize that the focus of those studies was the interaction between LH and the ovary; GnRH and LH pulse frequency were not controlled, ovaries were present, and the removal of the suckling stimulus affected LH concentrations and LH

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TABLE 1. Effect of experimental period on mean gonadotropin concentrations (least square means) in ovariectomized, GnRH-immunized gilts receiving GnRH-A pulses.[a]

Experimental period[a]	Mean LH (ng/ml)			Mean FSH(ng/ml)		
	Pre-treatment	Early treatment	Late treatment	Pre-treatment	Early treatment	Late treatment
First	0.48	0.94 (196)[b]	0.48 (100)	51.8	60.4 (117)	36.1 (70)
Second	0.25**	0.48** (192)	0.25** (100)	45.8	51.1* (112)	30.5 (67)
Third	0.21**	0.36** (171)	0.20** (95)	42.9	48.1* (112)	29.5 (69)
SEM	0.03	0.09	0.03	2.7	2.7	2.1

[a] Experimental design and protocols described in *Materials and Methods*; blood samples taken every 10 min for 6 h at the end of the 3-day pretreatment phase and at the beginning (early) and the end (late) of the 3-day treatment phase were used to calculate mean gonadotropin concentrations.
[b] Numbers in parentheses indicate percentage of pretreatment.
* Differs from value in first experimental period ($p \leq 0.04$).
** Differs from value in first experimental period ($p \leq 0.002$).

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pulse frequency. The focus of our study was the interaction between GnRH and LH/FSH; we controlled GnRH pulse frequency, and between-pig variation was eliminated through the experimental design. In our studies, increased GnRH pulse frequencies resulted in elevation of LH baseline concentrations bringing about increased mean LH concentrations. This validates Shaw and Foxcroft's conclusion [28] that there is a positive correlation between pulse frequency and minimum LH values, which may be a useful criterion on which to base estimates of LH activity during the follicular phase.

The greater LH response to acute stimulation with high-frequency GnRH pulses, as compared to the FSH response, indicates that greater amounts of releasable pools of LH were present and/or that the sudden increase of GnRH pulse frequency was a more appropriate stimulus for LH than for FSH release. Pituitary contents of LH and FSH decrease after hypothalamo-pituitary disconnection (HPD) in sheep [14] and after immunization against GnRH in rats [29], sheep [30], and pigs [31], but it has not been shown that FSH stores decrease more than LH stores. In the present experiment we also attempted to minimize and thereby equalize residual stores of LH and FSH by challenging gilts with a large dose of GnRH-A (Bolus1) in the beginning of each experimental period. Subsequently, we sought to stimulate gonadotropin synthesis by administration of GnRH-A pulses every 180 min for 3 days. Similarly, 250 ng GnRH administered every 2 h to ovariectomized, HPD ewes for 1 wk increased amounts of all three subunit mRNAs (LHβ, FSHβ, and α) [14], implying that LH and FSH synthesis can be stimulated with GnRH pulses. However, this pulsing regimen [14] resulted in increased pituitary content of LH, but not FSH, and this supports our view that releasable pools of LH were larger than those of FSH at the time frequent pulsing with GnRH was initiated. In addition, release of FSH into culture medium or the bloodstream is highly correlated with the degree of FSH synthesis [32–35], and most newly synthesized FSH subunits are assembled and released rather quickly [36–38]. In contrast, basal LH secretion is not tightly coupled to its synthesis [32], and a large portion of LH subunits are not immediately assembled and released after being synthesized but rather are retained in the endoplasmic reticulum [37, 39, 40] until final carbohydrate processing and subunit assembly is stimulated [41]. Our results are consistent with those observations; GnRH-stimulated release of LH occurred every 3 h during pretreatment, but basal release was minimal and newly synthesized LH was mostly stored. Resulting adequate intracellular stores were the basis of the pronounced increase of serum LH after stimulation with high-frequency GnRH-A pulses. In contrast, newly synthesized FSH was released quickly, and the marginal increase in mean FSH after initiation of high-frequency GnRH-A pulses implies a relative low amount of immediately releasable pools. Similarly, Clarke et al. [2] reported that short-term manipulations of GnRH frequency and amplitude had immediate and more definite effects on LH secretion than on FSH secretion in the HPD ewe, and that GnRH appeared to provide a trophic stimulus for FSH secretion, but that the direct secretory relationship that exists between GnRH and LH was not apparent for FSH. This implies that gonadal factors may provide the greatest regulatory influence on FSH and that GnRH is only supportive.

After 3 days of frequent pulsing with GnRH-A (30 or 60 min), serum FSH concentrations were suppressed compared to those seen with GnRH every 180 min. Likewise, in ovariectomized ewes that were desensitized by means of continuous GnRH infusion [42], serum FSH concentrations decreased below pretreatment concentrations. In contrast, LH release during late treatment was not different from the release of LH during pretreatment, apparently because the low-frequency GnRH-A stimulus during pretreatment al-

TABLE 2. Effect of experimental period on releasable pools of LH and FSH in ovariectomized, GnRH-immunized gilts, challenged with 5 μg GnRH-A (Bolus2).[a]

LH (AUC)[b]				FSH (AUC)[b]			
Experimental period				Experimental period			
First	Second	Third	SEM	First	Second	Third	SEM
399[c]	158[d]	165[d]	64	6650[c]	5471[c]	6710[c]	965

[a] Experimental design and protocols described in *Materials and Methods*.
[b] AUC (ng $\times$ ml^{-1} $\times$ min; least square means) during the 4-h sampling interval after challenge with 5 μg GnRH-A (Bolus2), i.v., at the end of each experimental period.
[c,d] Values identified by different letters differ ($p \leq 0.05$).

ready resulted in low LH. Dalkin et al. [9] reported that in castrated rats given testosterone replacement, a wide range of GnRH pulse frequencies (every 30–480 min; 25 ng/pulse) given for 24 h increased steady state concentrations of FSHβ mRNA but that maximal stimulation was associated with GnRH pulses given every 120 min. In the same model, transcription rate of FSHβ mRNA was only increased by GnRH pulses given every 120 min, not by higher GnRH pulse frequencies [11]. Other studies in rats [43], primates [12], and sheep [2] also suggest that FSH synthesis and secretion is favored by low-frequency GnRH pulses while higher-frequency stimuli favor LH, resulting in a decreased FSH:LH ratio. Our observations underline that low-frequency GnRH pulses are a very good stimulus for FSH synthesis and that the synthesized FSH was released quickly, resulting in high serum concentrations. Another indication that low-frequency pulses of GnRH-A were a better stimulator for FSH synthesis and release than for LH was provided by the fact that the FSH system was capable of recovering within 6 days from the effects of frequent GnRH-A pulsing and the subsequent large dose of GnRH-A (Bolus2). Before the next experimental period was initiated, gilts were not challenged with GnRH-A for 3 days; but then during the subsequent pretreatment phase, pituitaries were stimulated with GnRH-A pulses every 180 min for 3 days. This regimen resulted in restoration of mean serum FSH. In contrast to FSH, the LH system did not recover completely between experimental periods. This indicates that LH concentrations decreased during the resting phase and/or during the following pretreatment phase, and that LH synthesis was not optimally stimulated by GnRH-A pulses given every 180 min. Similarly, Kile et al. [44] reported that in ovariectomized, HPD ewes, low-frequency GnRH pulses (1 per 12 h) were more effective at restoring pituitary content of FSH than a pulse frequency of 1/h. Moreover, FSH-AUC after 5 μg GnRH-A (Bolus2) was not dependent on experimental period, but LH-AUC was lower in the second and third experimental period (Table 2). This confirms our conclusion that the FSH system undergoes a faster and more complete recovery during exposure to low-frequency GnRH-A pulses than the LH system.

Our results do not prove that low-frequency GnRH-A pulsing is the only factor responsible for maintaining FSH. It has been implied many times that factors other than GnRH may control FSH secretion [8, 14, 45–47]. Our experiments were not designed to evaluate the effects of activin, inhibin, follistatin, or of any other peptides, steroids, or neurotransmitters on gonadotropin release [3, 4, 48]. Especially activin of pituitary origin has been suggested as a stimulator of FSH [49–52]. It is conceivable that after ovariectomy, i.e., after the removal of the main source of inhibin and steroids—which negatively regulate FSH synthesis and release, activin may act uninhibited and as a cofactor to low pulse frequency of GnRH-A in keeping FSH at high levels during pulsing with GnRH-A every 180 min. Nevertheless, GnRH is ultimately necessary for continued FSH synthesis and release [22, 30, 53]. Clarke et al. [2] showed that FSH did not reappear in long-term (61–96 days) HPD ewes until 2 wk after pulsatile GnRH replacement, and Traywick and Esbenshade [22] were not able to reestablish detectable serum concentrations of FSH even after 144 h of administration of GnRH-A pulses to GnRH-immunized gilts. Hence, it seems that FSH release requires a certain history of GnRH exposure. That leads to the speculation that the putative cofactor of FSH release (possibly activin) is dependent on GnRH.

Effect of a Large Dose GnRH-A Stimulus (Bolus2)

The release of LH and FSH after the high terminal dose of GnRH-A (Bolus2) indicates that the pituitary glands were not depleted of gonadotropins, even though they were desensitized after treatment with high-frequency GnRH pulses. Desensitization is characterized by refractoriness of the pituitary gland to release LH and FSH in response to otherwise sufficient doses of GnRH; however, other studies have reported detectable levels of basal release [54] as we did in our study.

Validity of the Model and Appropriateness of GnRH-A

Serum gonadotropins were significantly lower 12 wk after immunization against GnRH than at Week 0, indicating that sufficient GnRH-antibodies were raised, but there may be some concern that gonadotropin concentrations were still detectable when treatments were initiated. In the ovariectomized/HPD sheep model [55], small pulses of GnRH that were administered for 2 days were able to sustain ongoing LH synthesis without release, and the LH response to large pulses administered on the third day was increased above the response obtained on Day 0. However, administration of small GnRH pulses during a large-pulse regimen did not alter mean LH release. Several other studies employing animal models that were actively immunized against GnRH also report low but detectable serum concentrations and pituitary concentrations of LH and FSH [22, 29–31, 56]. In our present experiments, possible low residual concentrations of endogenous GnRH were not expected to interfere with the aim of our study, because we controlled the primary GnRH frequency. Also, gonadotropin profiles that resulted from different treatments could be fully explained by the exogenous GnRH-A pulsing regimen and were independent of GnRH titers.

It is possible that the differences in LH response between the first and the second experimental period were based on larger pituitary pools of LH during the first experimental period despite our attempt to minimize the pools by administration of a large dose of GnRH-A. However, qualitative changes within experimental periods (rows of Latin rectangle) were very similar (Table 1). Moreover, the statistical model used to evaluate the effects of treatments accounted for variation introduced by differences among experimental periods and among pigs.

We used a potent GnRH agonist [57, 58]; however, this did not appear to be a problem in our present experiments or in earlier studies [18, 22]. Long-term pulsing (every 180 min for 6 days) in our present experiments did not diminish the pituitary's response to GnRH-A.

Our results demonstrate that a mere change in GnRH pulse frequency produces gonadotropin profiles that are consistent with gonadotropin changes observed during the reproductive cycle of the pig [1, 7, 19, 59]. During the luteal phase, a pattern dominated by low-frequency, high-amplitude LH pulses reflects low-frequency GnRH pulses resulting in low basal LH concentrations and high FSH concentrations, which is consistent with our observations during periods when GnRH-A pulses were applied every 180 min. During the follicular phase the pattern shifts to a high-frequency, low-amplitude pattern of LH release, indicating that high-frequency GnRH pulses are present, triggering increased synthesis of LH and decreased synthesis and release of FSH [1, 7, 24, 60]. Inhibin and estradiol are also elevated during this period and affect FSH negatively [3, 4], but our results show that high GnRH frequency alone

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would be capable of suppressing FSH secretion. The rapid and pronounced increase of LH in gilts that were pulsed with GnRH-A every 30 min in our study mimicked the occurrence of the LH surge, and our results imply that the LH surge is terminated because the pituitary gland becomes incapable of responding to an otherwise adequate stimulus, and not because of exhaustion of releasable LH pools.

The results of this study confirm that in the pig the response to altered GnRH-A pulse frequency differs between LH and FSH. High GnRH pulse frequency was more effective in releasing LH than in releasing FSH. Low GnRH pulse frequency supported FSH synthesis and release, but was not as effective in increasing LH concentrations.

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REFERENCES

1. van de Wiel DFM, Erkens J, Koops W, Vos E, Van Landeghem AAJ. Periestrous and midluteal time courses of circulating LH, FSH, prolactin, estradiol-17β and progesterone in the domestic pig. Biol Reprod 1981; 24:223–233.
2. Clarke IJ, Cummins JT, Findlay JK, Burman KJ, Doughton BW. Effects on plasma luteinizing hormone and follicle-stimulating hormone of varying the frequency and amplitude of gonadotropin-releasing hormone pulses in ovariectomized ewes with hypothalamo-pituitary disconnection. Neuroendocrinology 1984; 39:214–221.
3. Gregg DW, Schwall RH, Nett TM. Regulation of gonadotropin secretion and number of gonadotropin-releasing hormone receptors by inhibin, activin-A, and estradiol. Biol Reprod 1991; 44:725–732.
4. Ying S-Y. Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. Endocr Rev 1988; 9:267–293.
5. Wise ME. Gonadotropin-releasing hormone secretion during the postpartum anestrous period of the ewe. Biol Reprod 1990; 43:719–725.
6. Clarke IJ, Thomas GB, Yao B, Cummins JT. GnRH secretion throughout the ovine estrous cycle. Neuroendocrinology 1987; 46:82–88.
7. Flowers B, Cantley TC, Martin MJ, Day BN. Episodic secretion of gonadotrophins and ovarian steroids in jugular and utero-ovarian vein plasma during the follicular phase of the oestrous cycle in gilts. J Reprod Fertil 1991; 91:101–112.
8. Foxcroft GR, Shaw HJ, Hunter MG, Booth PJ, Lancaster RT. Relationships between luteinizing hormone, follicle-stimulating hormone and prolactin secretion and ovarian follicular development in the weaned sow. Biol Reprod 1987; 36:175–191.
9. Dalkin AC, Haisenleder DJ, Ortolano GA, Ellis TR, Marshall JC. The frequency of gonadotropin-releasing-hormone (GnRH) stimulation differentially regulates gonadotropin subunit messenger ribonucleic acid expression. Endocrinology 1989; 125:917–924.
10. Marshall JC, Griffin ML. The role of changing pulse frequency in the regulation of ovulation. Hum Reprod 1993; 8(suppl 2):57–61.
11. Haisenleder DJ, Dalkin AC, Ortolano GA, Marshall JC, Shupnik MA. A pulsatile gonadotropin-releasing hormone stimulus is required to increase transcription of the gonadotropin subunit genes: evidence for differential regulation of transcription by pulse frequency in vivo. Endocrinology 1991; 128:509–517.
12. Wildt L, Haeusler A, Marshall G, Hutchison JS, Plant TM, Belchetz PE, Knobil E. Frequency and amplitude of gonadotropin-releasing hormone stimulation and gonadotropin secretion in the Rhesus monkey. Endocrinology 1981; 109:376–385.
13. Kesner JS, Riebold TW, Convey EM. Effect of dosage and frequency of injection of luteinizing hormone releasing hormone on release of luteinizing hormone and follicle stimulating hormone in estradiol-treated steers. J Anim Sci 1982; 54:1023–1029.
14. Hamernik DL, Nett TM. Gonadotropin-releasing hormone increases the amount of messenger ribonucleic acid for gonadotropins in ovariectomized ewes after hypothalamic-pituitary disconnection. Endocrinology 1988; 122:959–966.
15. Fraser HM, Gunn A. Effects of antibodies to luteinizing hormone-releasing hormone in the male rabbit and on the rat oestrous cycle. Nature 1973; 244:160–161.
16. Esbenshade KL, Britt JH. Active immunization of gilts against gonadotropin-releasing hormone: effects on secretion of gonadotropins, reproductive function, and responses to agonists of gonadotropin-releasing hormone. Biol Reprod 1985; 33:569–577.
17. Fraser HM, Gunn A, Jeffcoate SL, Holland DT. Preparation of antisera to luteinizing hormone releasing hormone. J Endocrinol 1974; 61:ix-x.
18. Britt JH, Esbenshade KL, Ziecik AJ. Roles of estradiol and gonadotropin-releasing hormone in controlling negative and positive feedback associated with the luteinizing hormone surge in ovariectomized pigs. Biol Reprod 1991; 45:478–485.
19. Foxcroft GR, Van de Wiel DFM. Endocrine control of the oestrous cycle. In: Cole DJA, Foxcroft GR (eds.), Control of Pig Reproduction; Journal of Reproduction and Fertility Supplement. London: Butterworth Scientific; 1982: 161–177.
20. Clayton RN. Gonadotrophin-releasing hormone: its actions and receptors. J Endocrinol 1989; 120:11–19.
21. Nett TM, Crowder-Sousa ME, Herring RD, Hamernik DL. Effect of GnRH, progesterone, and estradiol on pituitary content of mRNAs for subunits of gonadotropins in sheep. In: Chin W, Boime I (eds.), Glycoprotein Hormones: Structure, Synthesis and Biologic Function. Serono Symposium. New York: Plenum Publ. Corp; 1990: 151–159.
22. Traywick GB, Esbenshade KL. Pulsatile administration of gonadotropin-releasing hormone agonist to gilts actively immunized against gonadotropin-releasing hormone. J Anim Sci 1988; 66:2209–2215.
23. Armstrong JD, Kraeling RR, Britt JH. Effects of naloxone or transient weaning on secretion of LH and prolactin in lactating sows. J Reprod Fertil 1988; 83:301–308.
24. Guthrie HD, Bolt DJ. Changes in plasma estrogen, luteinizing hormone, follicle-stimulating hormone and 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ during blockade of luteolysis in pigs after human chorionic gonadotropin treatment. J Anim Sci 1983; 57:993–1000.
25. SAS Institute Inc. SAS/STAT User's Guide, Release 6.03. Cary, NC: Statistical Analysis Systems Institute, Inc.; 1988: 549–640.
26. Veldhuis JD, Johnson ML. Cluster analysis: a simple, versatile, and robust algorithm for endocrine pulse detection. Am J Physiol 1986; 250(Endocrinol Metab 13):E486-E493.
27. Varley MA, Foxcroft GR. Endocrinology of the lactating and weaned sow. J Reprod Fertil Suppl 1990; 40:47–61.
28. Shaw HJ, Foxcroft GR. Relationships between LH, FSH and prolactin secretion and reproductive activity in the weaned sow. J Reprod Fertil 1985; 75:17–28.
29. Fraser HM, Gunn A, Jeffcoate SL, Holland DT. Effect of active immunization to luteinizing hormone releasing hormone on serum and pituitary gonadotrophins testes and accessory sex organs in the male rat. J Endocrinol 1974; 63:399–406.
30. Fraser HM, Clarke IJ, McNeilly AS. Effect of oestrogen and an LH-RH agonist on the release of gonadotrophins in ovariectomized ewes deprived of LH-RH. J Reprod Fertil 1981; 62:131–140.
31. Awoniyi CA, Chandrashekar V, Arthur RD, Schanbacher BD, Amador AG, Falvo RE. Pituitary and Leydig cell function in boars actively immunized against gonadotrophin-releasing hormone. J Reprod Fertil 1988; 84:295–302.
32. Chappel SC, Ulloa-Aguirre A, Coutifaris C. Biosynthesis and secretion of follicle-stimulating hormone. Endocr Rev 1983; 4:179–211.
33. Clayton RN. Regulation of gonadotrophin subunit gene expression. Hum Reprod 1993; 8(suppl 2):29–36.
34. Farnworth PG, Robertson DM, de Kretser DM, Burger HG. Effects of 31 kilodalton bovine inhibin on follicle-stimulating hormone and luteinizing hormone in rat pituitary cells in vitro: actions under basal conditions. Endocrinology 1988; 122:207–213.
35. Kitahara S, Winters SJ, Attardi B, Oshima H, Troen P. Effects of castration on luteinizing hormone and follicle-stimulating hormone secretion by pituitary cells from male rats. Endocrinology 1990; 126: 2642–2649.
36. Keene JL, Matzuk MM, Otani T, Fauser BCJM, Galway AB, Hsueh AJW, Boime I. Expression of biologically active human follitropin in Chinese hamster ovary cells. J Biol Chem 1989; 264:4769–4775.
37. Muyan M, Ryzmkiewicz DM, Boime I. Secretion of lutropin and follitropin from transfected GH_3 cells: evidence for separate secretory pathways. Mol Endocrinol 1994; 8:1789–1797.
38. Whitfield GK, Miller WL. Biosynthesis and secretion of follicle-stim-

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ulating hormone β-subunit from ovine pituitary cultures: effect of 17β-estradiol treatment. Endocrinology 1984; 115:154–159.

39. Corless CL, Matzuk MM, Ramabhadran TV, Krichevsky A, Boime I. Gonadotropin beta subunits determine the rate of assembly and the oligosaccharide processing of hormone dimer in transfected cells. J Cell Biol 1987; 104:1173–1181.
40. Kaetzel DM, Nilson JH. Methotrexate-induced amplification of the bovine lutropin genes in Chinese hamster ovary cells: relative concentration of the α and β subunits determines the extent of heterodimer assembly. J Biol Chem 1988; 263:6344–6351.
41. Muyan M, Baldwin DM. Testosterone suppresses 8-bromo-adenosine 3′,5′-monophosphate and gonadotropin-releasing hormone-stimulated luteinizing hormone subunit synthesis. Endocrinology 1992; 130: 3337–3344.
42. Nett TM, Crowder ME, Moss GE, Duello TM. GnRH-receptor interaction V. Down-regulation of pituitary receptors for GnRH in ovariectomized ewes by infusion of homologous hormone. Biol Reprod 1981; 24:1145–1155.
43. Haisenleder DJ, Katt JA, Ortolano GA, El-Gewely MR, Duncan JA, Dee C, Marshall JC. Influence of gonadotropin-releasing hormone pulse amplitude, frequency, and treatment duration on the regulation of luteinizing hormone (LH) subunit messenger ribonucleic acids and LH secretion. Mol Endocrinol 1988; 2:338–343.
44. Kile JP, Carnevali F, Nett TM. Restoration of pituitary content of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) after chronic exposure to estradiol requires different pulse frequencies of gonadotropin-releasing hormone (GnRH) stimulation. Biol Reprod 1992; 46(suppl 1):159 (abstract 436).
45. Clarke IJ, Burman KJ, Doughton BW, Cummins JT. Effects of constant infusion of gonadotrophin-releasing hormone in ovariectomized ewes with hypothalamo-pituitary disconnection: further evidence for differential control of LH and FSH secretion and the lack of a priming effect. J Endocrinol 1986; 111:43–49.
46. Culler MD, Negro-Villar A. Evidence that pulsatile follicle-stimulating hormone secretion is independent of endogenous luteinizing hormone-releasing hormone. Endocrinology 1986; 118:609–612.
47. Kartun K, Schwartz NB. Effects of a potent antagonist to gonadotropin-releasing hormone on male rats: luteinizing hormone is suppressed more than follicle-stimulating hormone. Biol Reprod 1987; 36:103–108.
48. Ying S-Y, Becker A, Baird A, Ling N, Ueno N, Esch F, Guillemin R. Type beta transforming growth factor (TGF-β) is a potent stimulator of the basal secretion of follicle stimulating hormone (FSH) in a pituitary monolayer system. Biochem Biophys Res Commun 1986; 135: 950–956.
49. Corrigan AZ, Bilezikhjan LM, Carroll RS, Bald LN, Schmelzer CH, Fendly BM, Mason AJ, Chin WW, Schwall RH, Vale W. Evidence for an autocrine role of activin B within rat anterior pituitary cultures. Endocrinology 1991; 128:1682–1684.
50. DePaolo LV, Mercado M, Guo Y, Ling N. Increased follistatin (activin-binding protein) gene expression in rat anterior pituitary tissue after ovariectomy may be mediated by pituitary activin. Endocrinology 1993; 132:2221–2228.
51. Kogawa K, Nakamura T, Sugino K, Takio K, Titani K, Sugino H. Activin-binding protein is present in pituitary. Endocrinology 1991; 128:1434–1440.
52. Meunier H, Rivier C, Evans RM, Vale W. Gonadal and extragonadal expression of inhibin α, βA, and βB subunits in various tissues predicts diverse functions. Proc Natl Acad Sci USA 1988; 85:247–251.
53. Rodin DA, Lalloz MRA, Clayton RN. Gonadotropin-releasing hormone regulates follicle-stimulating hormone β-subunit gene expression in the male rat. Endocrinology 1989; 125:1282–1289.
54. Badger TM, Loughlin JS, Naddaff PG. The luteinizing hormone-releasing hormone (LHRH)-desensitized rat pituitary: luteinizing hormone responsiveness to LHRH in vitro. Endocrinology 1983; 112: 793–799.
55. Clarke IJ, Cummins JT. The significance of small pulses of gonadotrophin-releasing hormone. J Endocrinol 1987; 113:413–418.
56. Jeffcoate IA, Keeling BJ. Active immunization against LH-RH in the female. In: Crighton DB (ed.), Immunological Aspects of Reproduction in Mammals; Proceedings of the 38th Nottingham Easter School in Agricultural Science (1983). London: Butterworths; 1984: 363–377.
57. Arimura A, Vilchez-Martinez JA, Coy DH, Coy EJ, Hirotsu Y, Schally AV. [d-Ala^6, Des-Gly-NH_2^{10}]-LH-RH-Ethylamide: a new analogue with unusually high LH-RH/FSH-RH activity. Endocrinology 1974; 95:1174–1177.
58. Karten MJ, Rivier JE. Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: rationale and perspective. Endocr Rev 1986; 7:44–66.
59. Hansel W, Convey EM. Physiology of the estrous cycle. J Anim Sci 1983; 57(suppl 2):404–424.
60. Prunier A, Martinat-Botté F, Ravault JP, Camous S. Periestrous patterns of circulating LH, FSH, prolactin and oestradiol–17β in the gilt. Anim Reprod Sci 1987; 14:205–218.

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